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Article

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Bioactivity-Guided Fractionation and GC/MS Fingerprinting of *Angelica sinensis* and *Angelica archangelica* Root Components for Antifungal and Mosquito Deterrent Activity

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Bioassay-guided fractionation of the chloroform extract from the roots of *Angelica sinensis* led to isolation and characterization of (*Z*)-ligustilide using direct-bioautography with *Colletotrichum* species. The structure of (*Z*)-ligustilide was confirmed by ^{1}H and ^{13}C NMR spectroscopy and GC/MS. (*Z*)-Ligustilide deterred the biting of two mosquito species more effectively than DEET. Three different *A. sinensis* accessions and one *Angelica archangelica* root oil were evauated by GC and GC/MS, and the dominant component in *A. sinensis* was 61−69% (*Z*)-ligustilide. Two other prominent compounds in *A. sinensis* oils were 5.7−9.8% (*E*)-3-butylidene phthalide and 1.5−2.3% (*Z*)-3-butylidene phthalide. The main constituents that comprised *A. archangelica* oil were monoterpene hydrocarbons such as 24.5% α-pinene, 13.8% δ-3-carene, 10.1% β-phellandrene, 8.8% *p*-cymene, 8.4% limonene, and 6.3% sabinene. Phthalides and monoterpene hydrocarbons were determined to be good systematic markers or chemical fingerprints for *A. sinensis* and *A. archangelica* root oils. Chemical fingerprinting by GC/MS of *A. sinensis* also confirmed the misidentification of one *A. archangelica* sample sold in the Chinese market.

KEYWORDS: (*Z*)-Ligustilide; dang gui; dong quai; *Colletotrichum acutatum*; *Colletotrichum fragariae*; *Colletotrichum gloeosporioides*; *Aedes aegypti*; *Anopheles stephensi*

INTRODUCTION

Plants used in traditional Chinese medicine (TCM) are commonly utilized directly or in dietary supplements throughout Asia. TCMs are traditionally prepared as teas, decoctions, or infusions by unique methodologies and in specific combinations of different herbs in the formulation (*1*). While the medicinal and functional aspects of TCM herbs have had some exploration as medicines, there has been little research to evaluate these plants as potential safe plant protectants. Our research at the U.S. Department of Agriculture, National Center for Natural Products Research, has several projects focusing on evaluating plants used in traditional medicines worldwide in the hope of finding new agrochemicals with exceptionally low mammalian and environmental toxicity (*2, 3*).

Angelica sinensis (Oliv.) Diels (Apiaceae) is an important medicinal herb in TCM. Dang gui (Chinese) or dong quai (English) is the Chinese name for the root of *Angelica sinensis* which has been used for more than 2000 years in China. Dong quai was adopted as the standardized common name in Herbs of Commerce (*4*). Use of Angelicae Radix was first recorded in the Divine Husbandman's Classic of the Materia Medica (*Shen Nong Bencao Jing*) in the first century B.C. (*4−6*). Historically, dong quai was used primarily as a general blood tonic and for gynecological disorders (*4, 6*). Two *Angelica* species are predominate in the literature. *Angelica archangelica* L. has been used in the treatment of gastric disorders (*7*). Phytochemically these species are very different and are often

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misidentified in the market and herbal shops and sold interchangeably and incorrectly. Therefore, we chose to investigate these two plants chemically and biologically in detail.

Recent therapeutic interest has focused on dong quai's cardiovascular, hematopoietic, hepatoprotective, antioxidant, antispasmodic, and immunomodulatory properties (*4*). The most active compounds in dong quai appear to be the phthalides, polysaccharides and ferulic acid (*4*). Phthalides are present in *A. sinensis* essential oils and used as an indicator of *A. sinensis* quality assessment and grading of plant material (*3, 5, 6, 8*). Its strong aromatic odor is related to the presence of (*Z*)-ligustilide, one of the major compounds in its essential oil, which is also an important marker used for assessing *A. sinensis* quality (*3, 5, 6, 8*). Preclinical studies indicate that dong quai and (*Z*)-ligustilide may also relax smooth muscle in the circulatory, respiratory and gastrointestinal systems (*4*). Phthalide derivatives may have potential as a new natural pesticide because recent research indicates that a large number of phthalides have insecticidal, herbicidal, nematicidal, antimicrobial and acaricidal activities (*9*).

A. archangelica has been used primarily as a digestive, as a tonic, for flatulence (*10*) and especially for mild cramping in gastrointestinal disturbances (*7*). The activity on smooth muscle may be the action that is common to both *A. sinensis* and *A. archangelica*. In folk medicine, *A. archangelica* is also used as an antiseptic, an expectorant, a diuretic and antiemetic (*11*). *A. archangelica* is used extensively in the liquor industry where it is used as a flavoring in liquor such as benedictine, boonekamp, and chartreuse (*7, 10, 11*).

The purpose of our reseach was to investigate the chemical composition of *A. sinensis* and *A. archangelica*, evaluate their GC and GC/MS phytochemical fingerprints, conduct bioassay-guided isolation, and identify biologically active compounds.

MATERIALS AND METHODS

Plant Material. *A. sinensis* (AS_1) root oil was obtained from Shaoyuan Chen, Zhejiang University, Hangzhou, China. *A. sinensis* roots (AS_2) were purchased as a plant material from Hsu's Ginseng Enterprises (Wisconsin), and its essential oil was extracted in our laboratory. Voucher specimens of AS_2 were deposited at the Herbarium of the Faculty of Pharmacy (ESSE 14427, Anadolu University, Eskisehir, Turkey). *A. archangelica* (AS_3) root oil was purchased from Shanghai Qika Corporation (Shanghai, China). *A. archangelica* root oil (AA) brought from China was purchased from Robertet Company (Shanghai, China).

General Experimental Procedures. *Hydrodistillation Process. A. sinensis* roots (AS_2) purchased from Ginseng Enterprises (Wisconsin) were water distilled for 3 h using a Clevenger-type apparatus (*12*) to produce an essential oil in 0.14% yield.

Extraction. A. sinensis (1.75 kg) roots were reduced into particles less than 6 mm and extracted with 8 L of 70% ethanol under 70 °C for 2 h twice. The liquid extract (approximately 15.5 L) was filtered with Whatman #4 filter paper and concentrated to a 1400 mL of ethanol-free aqueous extract under reduced pressure. A portion (100 mL) of the 1400 mL aqueous extract was freeze-dried to 55.4 g of crude residue (solid), and this yield was used to estimate the total crude residue of 775.6 g (ANS-C). The remaining aqueous extract (1300 mL containing an estimated 720.2 g of crude residue) was partitioned with chloroform (1:1; v/v) three times to obtain two fractions. The organic phase obtained with chloroform yielded ANS-CH (26.5 g) in solid block form and the water phase yielded ANS-WT (630 g) in powder form.

Chromatographic Conditions. GC/MS which was used to check the purity of (*Z*)-ligustilide was carried out at the National Center for Natural Products Research, The University of Mississippi. An HP 5890 series gas chromatography linked to an HP 5970 mass spectrometer system equipped with an HP automatic injector was used to check samples. The GC was equipped with a DB-1 capillary column (20 m × 0.18 mm i.d. with 0.25 μm film thickness). Helium (1 mL/min) was used as carrier gas. The GC oven temperature was kept at 70 °C for 3 min and programmed to 220 °C at a rate of 4 °C/min and then kept constant at 220 °C for 5 min to 240 °C at a rate of 1 °C/min. Mass range was recorded from *m/z* 40 to 550. MS were measured at 70 eV.

A. sinensis and *A. archangelica* oils were analyzed at the Department of Pharmacognosy, Anadolu University, Eskisehir, Turkey, by capillary GC-FID and GC/MS using an Agilent GC-MSD system under the chromatographic conditions as described below. Thin layer chromatography (TLC), normal particle precoated silica gel 60 F_{254} plates (Merck, Suwanee, GA); developing system, *n*-hexane:diethyl ether mixtures 95:5, 90:10, 85:15, 80:20, 70:30 v/v) using normal unsaturated chamber; visualization, vanillin/H_2SO_4 (1 g of vanillin in 100 mL of 20% H_2SO_4 in ethanol) and heat. High performance flash chromatography system (HPFC, Biotage, Inc., Horizon Pump, Charlottesville, Virginia), Biotage column Si 40 M (150 × 40 mm i.d.; 100 g KP-Sil silica; 40−63 μm particle size; flow rate, 5.0 mL/min).

Isolation of the Active Principles. Three extracts were then evaluated for their antifungal activity against three important plant pathogenic fungi *Colletotrichum* species using direct-bioautography. Only chloroform extract demonstrated nonselective activity against all three *Colletotrichum* species at 4 and 8 μL of 20 mg/mL. Bioassay-guided fractionation was used to sequentially purify the antifungal compounds against *Colletotrichum* species. Chloroform extract (1.5 g) was subjected to high performance flash chromatography system using a Biotage SI 40 M column (150 × 40 mm i.d.; 100 g KP-Sil silica; 40−63 μm particle size; flow rate, 5.0 mL/min) and eluted with *n*-hexane 100%, *n*-hexane−Et_2O mixtures 5, 20, 50, 80 (120 and 3.0 mL each eluent) and 100% EtOAc. Similar fractions according to TLC profiles (*n*-hexane: diethyl ether 95:5, 90:10, 85:15, 80:20, 70:30 v/v) were combined to give 7 pooled samples (fractions A1−A7, each 20 mL) that were subsequently subjected to antifungal testing using bioautography assays. Only fraction A4 demonstrated nonselective activity to all three *Colletotrichum* test species at 4 and 8 μL of 20 mg/mL. Fraction A4 was further evaluated by 1D-direct bioautography method using *n*-hexane−Et_2O (8:2, v/v) (Wedge et al. (*2*)) and demonstrated the presence of a single nonselective antifungal compound with an R_f value of 0.44. The antifungal compound (R_f = 0.44) was subsequently evaluated according to its TLC profile and then checked for purity using GC/MS. Fractions with a purity ≥99% were pooled and yielded 116 mg of pure compound, (*Z*)-ligustilide. This single compound was tested again against our three *Colletotrichum* species using bioautographic methods described subsequently.

GC-FID and GC/MS Conditions. A. sinensis and *A. archangelica* oils were analyzed by capillary GC and GC/MS using an Agilent GC-MSD system. The same column and analysis conditions were used for both GC and GC/MS. The GC/MS analysis was carried out with an Agilent 5975 GC-MSD system. HP-Innowax FSC column (60 m × 0.25 mm, 0.25 μm film thickness) was used with helium as carrier gas (0.8 mL/min). GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min, and kept constant at 220 °C for 10 min and then programmed to 240 °C at a rate of 1 °C/min, and kept constant at 240 °C for 20 min. Split ratio was adjusted at 40:1. The injector temperature was at 250 °C. MS were taken at 70 eV. Mass range was from *m/z* 35 to 450. The GC analysis was carried out using an Agilent 6890N GC system. FID detector temperature was 300 °C. In order to obtain same elution order with GC/MS, simultaneous injection was done by using the same column and appropriate operational conditions. Identification of the essential oil components were carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes (*13*−*15*). Computer matching against commercial (Wiley and MassFinder 3.1), and in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils, as well as MS literature data (*16*−*18*) was also used for the identification. The relative percentage amounts of the separated compounds were computed from FID chromatograms.

NMR Spectroscopy. The 1D NMR spectra were obtained on a Varian INOVA 600 at 600 (^1H) and 150 MHz (^{13}C). The chemical shift values are reported as parts per million (ppm) units relative to tetramethylsilane (TMS) for ^1H and ^{13}C. **(Z)-Ligustilide**: Yellowish oil. MS *m/z* (rel int):

190 $[M]^+$ for $C_{12}H_{14}O_2$ (67), 161 (100), 148 (86), 134 (26), 133 (19), 106 (41), 105 (55), 91 (14), 78 (30), 77 (19), 55 (48), 39 (7). ^{1}H NMR ($CDCl_3$): δ 6.23 (1H, dd, $J = 8.4$, 1.2 Hz, H-7), 5.95 (1H, dt, $J = 9.0$, 5.4 Hz, H-6), 5.18 (1H, t, $J = 7.8$ Hz, H-8), 2.54 (2H, t, $J = 9.6$ Hz, H_2-4), 2.40−2.43 (2H, m, H_2-5), 2.33 (2H, q, $J = 7.8$, 7.2 Hz, H_2-9), 1.46 (2H, q, $J = 7.2$, 6.6 Hz, H_2-10), 0.90 (3H, t, $J = 7.8$ Hz, H_3-11). ^{13}C NMR ($CDCl_3$): δ 167.9 (C-1), 148.8 (C-3a), 147.4 (C-3), 130.2 (C-6), 124.2 (C-7a), 117.2 (C-7), 113.2 (C-8), 29.3 (C-9), 22.6 (C-5, C-10), 18.7 (C-4), 14.0 (C-11). The spectral data were compared to the published data of (*Z*)-ligustilide by Miyazawa et al. (*19, 20*). The isomeric butylidenephthalides can be distinguished by the δH-8 values (*14*). δH-8 values were reported $\delta = 5.3$ ppm for the *Z*-isomer and 5.6 ppm for the *E*-isomer (*20*). Our result confirmed the δH-8 value of 5.18 for *Z*-isomer.

Direct Bioautography Assay for Activity against Plant Pathogenic Fungi. Pathogen production and bioautography procedures of Tabanca et al. (*21*), Meazza et al. (*22*) and Fokialakis et al. (*23*) were used to evaluate antifungal activity against fungal plant pathogens. Sensitivity of each fungal species to each test compound was determined 4 days after treatment by comparing the size of inhibitory zones. Means and standard deviations of inhibitory zone size were used to evaluate antifungal activity of fractions and test compounds. Bioautography experiments were performed multiple times using both dose- and nondose−response formats. Technical grade commercial fungicides benomyl, cyprodinil, azoxystrobin, and captan (Chem Service, Inc., West Chester, PA) were used as fungicide standards at 2 mM in 2 μL of 95% ethanol. (*Z*)-Ligustilide was applied at 4 and 8 μL of 2 mM concentration in *n*-hexane. *Angelica* essential oils and chloroform extracts and fractions were applied at 4 and 8 μL of 20 mg/mL of *n*-hexane on to TLC plates. Antifungal activity can be visualized directly on the TLC plate as "clear zones" where no fungal mycelia, stroma, or condia grow (*24*). Zones with "diffuse inhibition" are "growth suppressive" in nature and mycelia, stroma or condia growth on at a reduced level. Fungal growth inhibition means for compounds and essential oils were analyzed separately by ANOVA using SAS software, Ver. 8. Mean separations were performed based on Fisher's protected least significant difference (LSD) ($P = 0.05$). Statistical comparisons were made for fungal growth across compounds and of compound across fungal growth.

Mosquito Bioassay. Aedes aegypti (L.) (red eye Liverpool strain) and *Anopheles stephensi* Liston (Delhi strain) used in the study were from colonies maintained at the Walter Reed Army Institute of Research, Department of Entomology, Silver Spring, MD. The mosquitoes were reared using the procedure of Gerberg et al. (*25*). Larvae were fed ground tropical fish flakes (Tetramin Tropical Fish Flakes, Tetra Sales, Blacksburg, VA, www.tetra-fish.com). Colonies were held in a photoperiod of 12:12 h (L:D with lights on at 0600 h) at 27 °C and 80% RH. Adult mosquitoes were fed using cotton pads moistened with 10% aqueous sucrose solution. Mated nulliparous *Ae. aegypti* and *An. stephensi* females (5−15 d old) were used in the testing. *An. stephensi* had access to water only 24 h before testing, and *Ae. aegypti* had no water 24 h before testing.

The comparative mosquito feeding-deterrent activity of (*Z*)-ligustilide, callicarpenal, and *N*,*N*-diethyl-3-methylbenzamide (DEET) was evaluated using the two species of mosquitoes. (*Z*)-Ligustilide was prepared as described in this paper. Pure callicarpenal was isolated from *Callicarpa* species (*26*). Callicarpenal was selected for testing because previous study of this plant-derived compound showed that it was a potent deterrent of mosquito biting (*26*). DEET (*27*) was purchased from Morflex, Inc., Greensboro, NC. DEET is considered to be the best mosquito repellent ever developed (*28*) and is often used as a golden standard to which new candidate repellents are compared. Bioassay of the compounds was conducted by using an in vitro Klun and Debboun (K&D) module bioassay system (*29*). A bioassay replicate consisted of four randomized treatments of (*Z*)-ligustilide, callicarpenal, DEET applied to organdy cloth in ethanol and ethanol-treated cloth as control. A 25 nmol compound/cm^2 cloth dose was used in all tests because previous bioassays with DEET (*29, 30*) consistently showed 80% suppression of mosquito biting compared to control.

The bioassays were conducted with the in vitro K&D module system positioned in a PURAIR ductless chemical fume hood (Air Science USA LLC, Fort Myers, FL), and tests were conducted from 1300 to 1600 h at 24−26 °C and 24−51% RH in ambient laboratory light over several days.

In replicated tests, each of the four chemical treatments was tested against 85 *Ae. aegypti* females (17 replicates) and 100 female *An. stephensi* (20 replicates), respectively, and the proportion of mosquitoes not biting for each treatment was calculated. For each experiment, a logistic regression approach (*30, 31*) was used to model the proportion of nonbiting mosquitoes jointly for each group of compounds, including the control. Our statistical testing was done in a logistic regression framework using dummy variables (contrasts with the control or contrasts with DEET). Nonbiting proportions were converted to logits ($\log(p/(1-p))$), and contrasts with the control and DEET were tested using *t* tests. This statistical approach is more powerful than the more traditional way of converting the proportions using the arcsine transformation and then running an analysis of variance. Because the data were binomial (count of number of mosquitoes biting out of total number tested), a measure of variability came directly from the binomial distribution. As example, a standard error for the proportion not biting is $SE = \sqrt{[p(1-p)/n]}$ where p is the proportion not biting and n is the number of mosquitoes tested. The level of significance was set at $P = 0.05$.

RESULTS AND DISCUSSION

Essential oils from the roots of three different *A. sinensis* collections (AS_1, AS_2 and AS_3) and one *A. archangelica* (AA) were characterized and identified by gas chromatography and gas chromatography/mass spectrometry. Characterized compounds reported with their relative percentages are listed in **Table 1**. Forty (AS_1), 49 (AS_2), and 40 (AS_3) compounds were identified from *A. sinensis* essential oils that constituted 96.9%, 95.1% and 94.7% of the total oil, respectively. *A. sinensis* oils are characterized as the major compound by high contents of *Z*-ligustilide (*4*). The oils of AS_1, AS_2 and AS_3 in our study were characterized by the presence of (*Z*)-ligustilide at 60.9%, 69.2% and 68.2%, respectively. (*E*)-3-Butylidene phthalide (AS_1, 5.7%; AS_2, 9.8%; AS_3, 6.4%) and (*Z*)-3-butylidene phthalide (AS_1, 1.5%; AS_2, 2.3%; AS_3, 1.7%) were the two prominent compounds in *A sinensis*.

The largest part of the essential oil from *A. archangelica* L. roots was composed of monoterpene hydrocarbons (*7, 11, 32, 33*). α-Pinene was found as a dominant constituent in more than half of the investigated plant oils obtained from Finland, Norway, France, and Brazil (*33*). Other dominant components such as β-phellandrene, δ-3-carene, and β-pinene, limonene, *p*-cymene and α-phellandrene were also found in the *A. archangelica* oils (*33*). The majority of essential components we found in *A. archangelica* root oil corroborated the reports above and were determined to be monoterpene hydrocarbons: α-pinene (24.5%), δ-3-carene (13.8%), β-phellandrene (10.1%), *p*-cymene (8.8%), limonene (8.4%) and sabinene (6.3%) (**Table 1**). Forty-five compounds were identified and comprised 92.4% of the total oil. The importance and usefulness of GC/MS chemical fingerprinting during this study demonstrated that one sample of *A. sinensis* was misidentified as *A. archangelica* by the vendor in China. Our GC/MS profile indicated that the *A. sinensis* oil was rich in phthalides not in monoterpene hydrocarbons and was misidentified and was not *A. archangelica* as originally indicated (**Figure 1**). After inquiry into exact identification with the supplier the company confirmed that the *A. archangelica* was actually *A. sinensis.*

Angelica oils were evaluated for antifungal activity against three *Colletotrichum* species using our direct-bioautography assays. Three *A. sinensis* oils showed antifungal activity at 20 mg/mL, using a 4 and 8 μL test volume, against *Colletotrichum acutatum*, *C. fragariae*, *C. gloesporides* and were compared with

Table 1. The Composition of the Essential Oils of *A. sinensis* and *A. archangelica*[a]

		composition, %						composition, %			
RRI	compound	AS_1	AS_2	AS_3	AA	RRI	compound	AS_1	AS_2	AS_3	AA
1032	α-pinene	1.7	4.6	tr	24.5	1668	(*Z*)-β-farnesene	0.2	0.1	0.2	tr
1035	α-thujene	–	–	–	1.3	1674	muurola-4,11-diene	0.4	0.1	0.3	–
1076	camphene	–	–	tr	0.9	1687	α-humulene	–	–	–	0.4
1093	hexanal	–	0.2	tr	–	1690	cryptone	–	–	–	1.1
1100	undecane	0.3	0.1	tr	–	1706	α-terpineol	–	0.1	–	–
1118	β-pinene	tr	0.1	–	1.4	1708	ledene	0.3	–	–	–
1132	sabinene	–	–	–	6.3	1722	dodecanal	–	0.1	–	–
1135	thuja-2,4(10)-diene	–	–	–	0.5	1726	β-chamigrene	0.2	0.1	0.2	–
1146	δ-2-carene	–	–	–	0.1	1726	germacrene D	–	–	–	0.1
1159	δ-3-carene	–	–	–	13.8	1740	α-muurolene	–	–	–	0.1
1174	myrcene	0.1	tr	tr	4.8	1741	β-bisabolene	0.7	0.3	0.6	–
1176	α-phellandrene	–	–	–	1.7	1747	α-alaskene	0.1	tr	0.2	–
1203	limonene	tr	tr	tr	8.4	1755	bicyclogermacrene	0.1	tr	0.2	–
1205	sylvestrene	–	–	–	0.2	1758	*cis*-piperitol	–	–	–	0.1
1218	β-phellandrene	–	–	–	10.1	1759	α-cuprenene	0.1	tr	0.1	–
1246	(*Z*)-β-ocimene	16.7	1.4	0.8	0.8	1773	δ-cadinene	–	–	–	0.1
1255	γ-terpinene	–	tr	tr	0.4	1783	β-sesquiphellandrene	0.1	tr	0.2	–
1266	(*E*)-β-ocimene	0.2	tr	tr	1.7	1801	β-cuprenene	0.2	0.1	0.2	–
1278	*m*-cymene	–	–	–	0.1	1823	*p*-mentha-1(7),5-dien-2-ol	–	–	–	0.3
1280	*p*-cymene	–	tr	tr	8.8	1849	cuparene	0.4	0.2	0.2	–
1286	isoterpinolene	–	–	–	0.3	1864	*p*-cymen-8-ol	–	–	–	0.2
1290	terpinolene	–	tr	–	0.3	1878	guaiacol	–	–	0.1	–
1382	*cis*-alloocimene	0.5	–	–	–	1973	dodecanol	–	0.1	tr	–
1384	α-pinene oxide	–	–	–	0.1	2019	2,3,6-trimethylbenzaldehyde	0.2	1.1	0.1	–
1400	tetradecane	–	0.1	tr	–	2041	pentadecanal	–	0.1	–	–
1402	(*E*,*Z*)-1,3,5-undecatriene	–	–	–	0.1	2098	globulol	0.1	0.1	–	–
1409	*trans*-alloocimene	0.2	–	–	–	2104	viridiflorol	0.1	0.1	–	–
1413	rose furan	tr	–	–	0.1	2131	hexahydrofarnesyl acetone	–	–	0.4	–
1426	pentyl benzene	0.2	0.1	tr	–	2144	rosifoliol	–	0.1	–	–
1452	α,*p*-dimethylstyrene	–	–	–	0.1	2144	spathulenol	1.9	0.7	0.6	–
1458	*cis*-1,2-limonene epoxide	–	–	–	0.1	2157	*cis*-*p*-menth-4-ene-1,2-diol	–	–	–	0.2
1477	4,8-epoxyterpinolene	–	–	–	0.1	2179	tetradecanol	–	0.1	–	–
1497	α-copaene	–	–	–	0.4	2218	4-vinyl guaiacol	0.3	0.4	0.9	–
1553	linalool	–	–	–	0.1	2226	methyl hexadecanoate (=methyl palmitate)	–	0.1	0.1	–
1571	*trans*-*p*-menth-2-en-1-ol	–	–	–	0.1	2247	*trans*-α-bergamotol	0.3	0.1	0.1	–
1587	β-funebrene	0.4	0.1	–	–	2287	1-pentadecanol	0.2	0.3	0.2	–
1591	bornyl acetate	–	–	–	0.4	2304	14-pentadecanolide	–	–	–	0.4
1591	1,7-diepi-β-cedrene	–	0.1	0.2	–	2384	1-hexadecanol	0.1	–	–	–
1600	β-elemene	–	–	–	0.1	2530	sedecanoic acid lactone	0.8	0.1	–	–
1611	terpinen-4-ol	–	tr	–	1.0	2554	(*E*)-3-butylidene phthalide	5.7	9.8	6.4	–
1613	β-cedrene	0.1	–	–	–	2557	3-*n*-butyl phthalide	tr	tr	–	–
1628	aromadendrene	0.2	tr	0.1	–	2609	(*Z*)-3-butylidene-3,4-dihydro phthalide (=(*Z*)-ligustilide)	60.9	69.2	68.2	–
1638	*cis*-*p*-menth-2-en-1-ol	–	–	–	0.1	2651	(*Z*)-3-butylidene phthalide	1.5	2.3	1.7	–
1644	widdrene (=thujopsene)	0.1	tr	tr	–	2900	nonacosane	–	–	2.0	–
1658	sabinyl acetate	–	–	–	0.2	2931	hexadecanoic acid	0.6	2.1	9.9	–
1667	β-barbatene	0.7	0.4	0.5	–		total	96.9	95.1	94.7	92.4

[a] AS_1: *A. sinensis* oil from Shaoyuan Chen, Zhejiang University, Hangzhou, China. AS_2: *A. sinensis* from Hsu's Ginseng Enterprises (Wisconsin). AS_3: *A. sinensis* oil from Shanghai Qika Corporation, Shanghai, China. AA: *A. archangelica* oil from Robertet Company, Shanghai, China. RRI: Relative retention indices calculated against *n*-alkanes. Relative percentage calculated from FID data using a HP-Innowax column. tr: Trace (< 0.1%).

commercial fungicide standards (**Table 2**). *Angelica archangelica* root oil showed no antifungal activity against our three *Colletotrichum* test species. We also evaluated crude, aqueous, and chloroform extracts of *A. sinensis* roots at 4 and 8 μL of 20 mg/mL concentration using the *Colletotrichum* direct-bioautography assay. Water and crude extracts were not active. The chloroform fraction was identical to the three *A. sinensis* essential oils under the same TLC conditions (*n*-hexane−Et_2O 8:2, v/v). Subsequent direct bioautography of the TLC plates indicated that antifungal activity and the R_f value of 0.44 were the same for the chloroform extract and three *A. sinensis* essential oils. Bioassay-guided isolation studies were performed on chloroform extract and resulted in the isolation of compound, (*Z*)-ligustilide (**Figure 2**). ^1H and ^{13}C NMR spectroscopic data compared with literature values (*19, 20*).

Pure (*Z*)-ligustilide was subsequently spotted onto the TLC plate at 4 and 8 μL of a 2 mM concentration using the direct bioautography assay and demonstrated moderate antifungal activity against all three *Colletotrichum* species (**Table 2**). The three *A. sinensis* essential oils demonstrated higher levels of nonspecific antifungal activity to *Colletotrichum* species than did (*Z*)-ligustilide. The chloroform extract (ANS-CH) showed the same level of activity as the original essential oil which confirmed the TLC results. The four commercial fungicide standards demonstrated levels of antifungal activity or low levels of diffuse inhibitory zone depending on their mode of action that were consistent with the sensitivity/resistance profile for each fungal isolate. We previously reported that (*Z*)-ligustilide showed moderate activity in a 96-well microdilution broth assay against *Botrytis cinerea* and further demonstrated phytotoxicity and antialgal activities of this compound, (*Z*)-ligustilide (*34*).

Because phthalide derivatives have a history of insecticidal activity, we also evaluated (*Z*)-ligustilide for antimosquito activity. **Table 3** reports the results of mosquito bioassays using 25 nmol compound/cm^2 cloth doses of (*Z*)-ligustilide, callicarpenal, and DEET compared to control with *A. aegypti* and

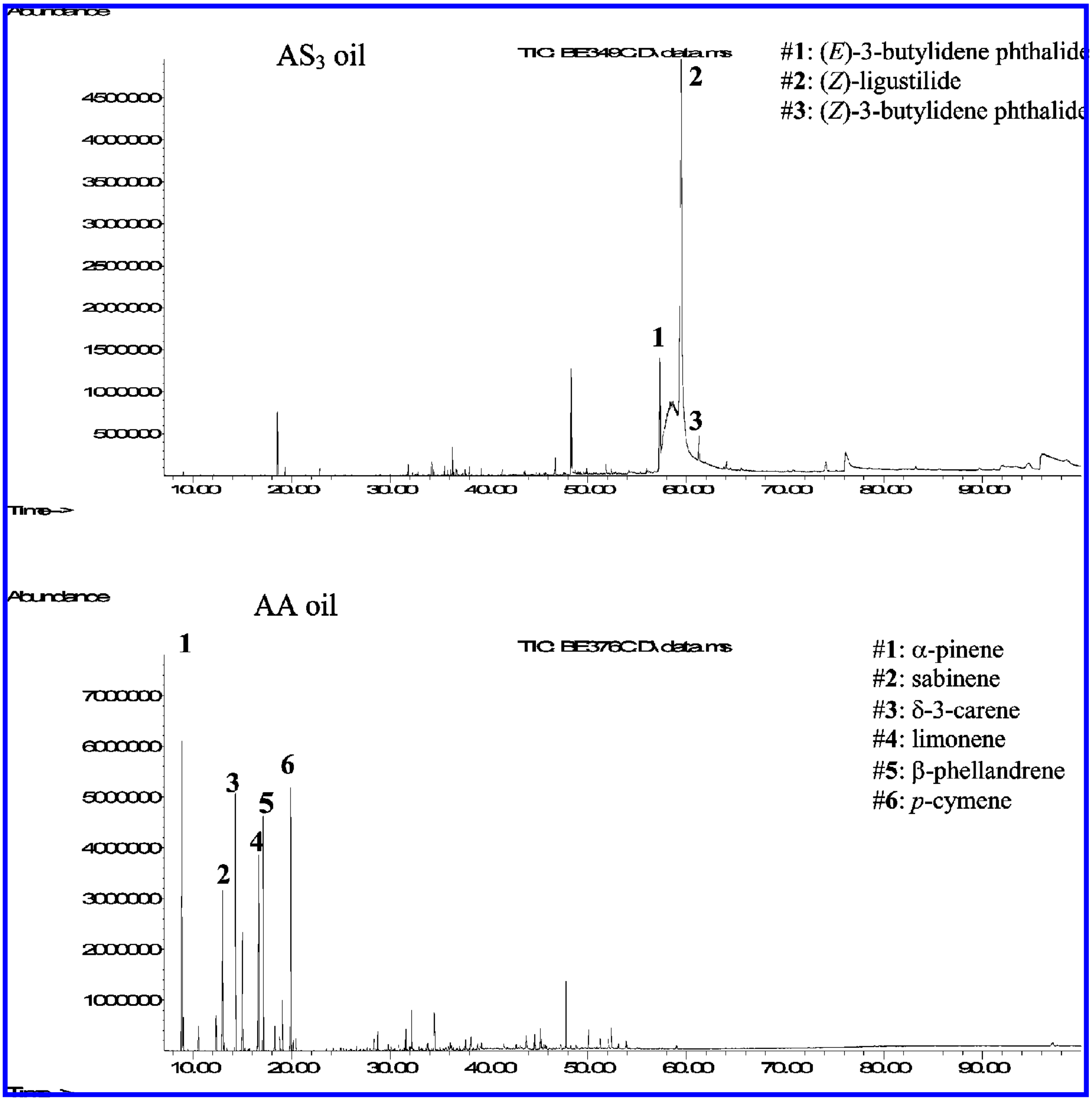

Figure 1. GC/MS fingerprint chromatogram of *A. sinensis* (AS_3) and *A. archangelica,* root oils. GC/MS chromatograms were obtained with a HP-Innowax column.

An. stephensi. The proportion of mosquitoes not biting data show that the three compounds were significantly more effective than control ethanol-treated cloth in deterring biting. (*Z*)-Ligustilide and callicarpenal deterred the biting of both mosquito species more effectively than DEET. This evidences an outstanding performance of the plant derived compounds over DEET. (*Z*)-Ligustilide is known from previous studies to exhibit a wide range of biological activities that include insecticidal, phytotoxic, antifungal, antiviral, and antimicrobial properties (*19, 35*), and we now report that it is also a potent deterrent of mosquito feeding. Dethier et al. (*36*) defined a deterrent as a chemical that inhibits feeding when present in a place where insects would, in its absence, normally feed. Thus, the in vitro K&D module system used in this study specifically quantified the mosquito feeding-deterrent properties of (*Z*)-ligustilide. Klun et al. (*37*) demonstrated that chemicals having feeding deterrent effects can, at the same time, also possess repellent effects. Thus, in some other bioassay modality (*Z*)-ligustilide might prove to be a repellent which is defined (*36*) as a chemical that causes insects to make oriented movement away from its source.

There are many different botanicals named under dong quai or dang gui and sold in the herbal trade interchangeably. Suitable methods of chemical analysis exist for phytochemical fingerprinting dong quais to confirm proper botanical identity and distinguish from *A. acutiloba* Siebold. & Zucc. (dong dang qui), *A. megaphylla* Diels. (da ye dang qui), *A. valida* Diels. (Jin Shan dang qui), *A. gigas* Nakai (Chaoxinan dang qui or Chamdang qui), *A. uchiyamana* Yabe and *Ligusticum glaucescens* Franch. (ye dang qui) (*4*). The importance and usefulness of GC/MS chemical fingerprinting during this study demonstrated that plant species purchased even from reputable suppliers are misidentified and chemical techniques are necessary to confirm identity of experimental samples.

Alkylphthalides and monoterpene hydrocarbons are considered as systematic markers or chemical fingerprints for *A. sinensis* and *A. archangelica* root oils. (*Z*)-Ligustilide showed moderate antifungal activity against anthracnose causing fungi that infect numerous foliage and fruit crops worldwide. The benefits of using essential oils as agrochemicals are many. Essential oils with antifungal activity often possess very low

Table 2. Antifungal Activity of *Angelica* Essential Oils and *A. sinensis* Extracts Using Direct Bioautography against Three *Colletotrichum* Species[a]

samples	mean fungal growth inhibition[§] (mm) ± SD					
	C. acutatum		*C. fragariae*		*C. gloeosporioides*	
	4 μL	8 μL	4 μL	8 μL	4 μL	8 μL
AS_1	6.5 ± 0.71a	10.0 ± 0.0	8.5 ± 0.71	10.5 ± 0.71	8.5 ± 0.71	10.5 ± 0.71
AS_2	5.5 ± 0.71ab	9.5 ± 0.71	6.0 ± 0.71	10.5 ± 0.71	5.5 ± 0.71	9.5 ± 0.71
AS_3	6.5 ± 0.71a	10.5 ± 0.71	7.0 ± 0.0	11.0 ± 0.0	6.5 ± 0.71	10.5 ± 0.71
AA	0 ± 0c	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
ANS-C	0 ± 0c	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
ANS-CH	6.5 ± 0.71a	9.5 ± 0.71	7.5 ± 0.71	11.5 ± 0.71	6.5 ± 0.71	11.0 ± 0.0
ANS-WT	0 ± 0c	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
(*Z*)-ligustilide	4.5 ± 0.71b	6.5 ± 0.71	5.5 ± 0.71	7.5 ± 0.71	5.0 ± 0.0	7.0 ± 0.0
LSD	1.289	1.153	0.9985	1.153	1.153	0.9985
agrochemical standards	2 μL		2 μL		2 μL	
benomyl*	Dz		21.3 ± 0.35		Dz	
captan*	11.5 ± 0.71		15.0 ± 0.49		18.9 ± 1.41	
cyprodinil*	Dz		Dz		Dz	
azoxystobin*	Dz		26 ± 1.41		Dz	

[a] AS_1: *A. sinensis* oil from Shaoyuan Chen, Zhejiang University, Hangzhou, China, AS_2: *A. sinensis* from Hsu's Ginseng Enterprises (Wisconsin). AS_3: *A. sinensis* oil from Shanghai Qika Corporation, Shanghai, China. AA: *A. archangelica* oil from Robertet Company, Shanghai, China. ANS-C: crude extract of *A. sinensis* roots. ANS-CH: Chloroform extract of A. *sinensis* roots. ANS-W: Aqueous extract of *A. sinensis* roots. Essential oils were applied at 4 and 8 μL of a 20 mg/mL solution onto a silica TLC plate. [§]Mean inhibitory zones and standard deviations (SD) were used to determine the level of antifungal activity against each fungal species. *Technical grade agrochemical fungicides (without formulation) with different modes of action were used as internal standards at 2 mM in 2 μL. Dz: Diffuse inhibitory zone. Mean values followed by different letters within column (lower case, by fungus) and within the row (upper case, by chemical) are significantly (P = 0.05) different as determined by LSD.

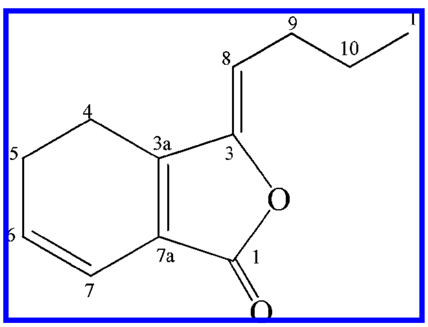

Figure 2. Structure of compound, (*Z*)- ligustilide.

Table 3. Mosquito Feeding Deterrent Activity of (*Z*)-Ligustilide Compared to DEET and Callicarpenal, Each Applied to Cloth at 25 nmol compound/cm^2 cloth, and a Control[a]

treatment	proportion of mosquitoes not biting (std error)	
	Aedes aegypti	*Anopheles stephensi*
control	0.21 (0.04) a	0.26 (0.04) a
(*Z*)-ligustilide	0.79 (0.04) bc	0.66 (0.04) bc
callicarpenal	0.91 (0.03) bc	0.72 (0.04) bc
DEET	0.67 (0.05) b	0.51 (0.04) b

[a] Each treatment was tested against 85 *Aedes* and 100 *Anopheles* females. a = Not different from control. b = Significantly different from control. c = Significantly different from DEET. P = 0.05.

levels of phytotoxicity to the plant and are often much less toxic than classical horticultural oils. The lipophilic nature of essential oils allows their constituents to embed themselves into the leaf waxes and persist through rainfall, hence making them potentially useful agrochemicals. Natural antimicrobial agents from plants, in their combined form as essential oil or as individual components, are generally broad-spectrum compounds with low mammalian and environmental toxicity. The broad spectrum activity and oily nature also make them good candidates as mosquito deterrents. The issue of highest concern that (*Z*)-ligustilide has is stability. (*Z*)-Ligustilide is unstable in air and requires refrigeration and is best stored under nitrogen at 4 °C in an amber vial. However, like strobilurin fungicides modifications to an active pharmacophore can lead to a multimillion dollar class of new agrochemicals.

(*Z*)-Ligustilide, like the previously identified natural-product mosquito feeding deterrent compound, callicarpenal, is a new lead compound that represents an alternative to traditional synthetic chemicals that have been developed and used for protection against blood-feeding arthropods that vector human diseases. The in vitro K&D assay method provided a snapshot of (*Z*)-ligustilide's mosquito feeding deterrent activity in a three minute exposure to the mosquitoes. This result does little to indicate what utility the compound may have from a practical standpoint. For a compound to effectively serve as a personal protection chemical it should retain protective activity for at least six hours after application to clothing or skin. Most natural products on the market today lack adequate duration of activity as compared to DEET (*38*), which is known to provide up to six hours of protection postapplication. Thus, to bring a compound like (*Z*)-ligustilide to the market as an agrochemical or mosquito deterrent, major hurdles in product development would include the design of formulation technology to give long duration activity for the compound, toxicological testing to demonstrate safety for human use, and U.S. Environmental Protection Agency registration.

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